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Histone Modification in Constitutive Heterochromatin Versus Unexpressed Euchromatin in Human Cells

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Abstract Histone modifications are implicated in regulating chromatin condensation but it is unclear how they differ between constitutive heterochromatin and unexpressed euchromatin. Chromatin immunoprecipitation (ChIP) assays were done on various human cell populations using antibodies specific for acetylated or methylated forms of histone H3 or H4. Analysis of the immunoprecipitates was by quantitative real-time PCR or semi-quantitative PCR (SQ-PCR). Of eight tested antibodies, the one for histone H4 acetylated at lysine 4, 8, 12, or 16 was best for distinguishing constitutive heterochromatin from unexpressed euchromatin, but differences in the extent of immunoprecipitation of these two types of chromatin were only modest, although highly reproducible. With this antibody, there was an average of 2.5-fold less immunoprecipitation of three constitutive heterochromatin regions than of four unexpressed euchromatic gene regions and about 15-fold less immunoprecipitation of these heterochromatin standards than of two constitutively expressed gene standards ($P < 0.001$). We also analyzed histone acetylation and methylation by immunocytochemistry with antibodies to H4 acetylated at lysine 8, H3 trimethylated at lysine 9, and H3 methylated at lysine 4. In addition, immunocytochemical analysis was done with an antibody to heterochromatin protein 1α (HP1α), whose preferential binding to heterochromatin has been linked to trimethylation of H3 at lysine 9. Our combined ChIP and immunocytochemical results suggest that factors other than hypoacetylation of the N-terminal tails of H4 and hypermethylation of H3 at lysine 9 can play an important role in determining whether a chromatin sequence in mammalian cells is constitutively heterochromatic. J. Cell. Biochem. 93: 286–300, 2004.

Key words: heterochromatin; histone acetylation; histone methylation; ChIP; immuno-FISH

Interest in constitutive heterochromatin has been increasing lately, not only because of its essential role as a structural determinant of chromosomes in most eucaryotes [Ekwall et al., 1997; Bernard et al., 2001; Peters et al., 2001; Sullivan et al., 2001], but also because it can be a mediator of the control of expression of certain genes in *cis* and others in *trans* [Talbert and Henikoff, 2000; Gasser, 2001; Matsuda et al., 2001; Schotta et al., 2002]. Furthermore, understanding how the highly condensed state of constitutive heterochromatin is established and maintained can give insights into the related phenomenon of transcription repression-associated condensation of euchromatin, often referred to as heterochromatinization. This condensation can be either localized at euchromatic promoter regions or spread over large gene regions, and it is often linked to modification of the core histones [Gilbert and Sharp, 1999; Eberharter and Becker, 2002]. The histone modification-sensitive binding of heterochromatin protein 1 (HP1) family members to certain inactive euchromatic gene regions [Nielsen et al., 1999; Hwang et al., 2001; Li et al., 2002] as well as to constitutive heterochromatin [Belyaeva et al., 1993; Minc et al., 1999; Richards and Elgin, 2002] illustrate interrelationships between these two kinds of chromatin. In *Drosophila*, mammalian cells, and fission yeast, HP1-type proteins participate in position-effect variegation (PEV), which is the epigenetically inherited repression of euchromatic genes that are abnormally positioned near constitutive heterochromatin or that

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become multimerized and, presumably, heterochromatic [Festenstein et al., 1999; Grewal and Elgin, 2002]. Histone H3 methylation at lysine 9 (H3 Me K9) has been linked to HP1 binding and heterochromatinization in PEV or in normal gene repression. In contrast, hyperacetylation of the N-terminal tails of core histones and hypermethylation of H3 at lysine 4 are characteristic of expressed gene or promoter regions [Coffee et al., 1999; Gilbert and Sharp, 1999; Hwang et al., 2001; Litt et al., 2001; Noma et al., 2001; Li et al., 2002].

Constitutive heterochromatin in vertebrate cells is defined cytologically as the chromatin that remains highly condensed in interphase irrespective of cell type. Unexpressed euchromatin, such as gene regions not transcribed in a given cell type, show lower extents of nuclease sensitivity and histone acetylation than actively transcribed euchromatic regions [Bulger et al., 2003]. Only a few reports have described differences between the local compaction of constitutive heterochromatin and unexpressed euchromatin in interphase cells of higher eucaryotes. Nucleosomes containing variegating transgenes inserted into constitutive heterochromatin of *Drosophila* have an unusually regular spacing [Wallrath and Elgin, 1995; Sun et al., 2001], as observed for fission yeast centromeres [Allshire et al., 1994]. Wallrath and Elgin found a decrease of up to tenfold in the restriction endonuclease sensitivity of an uninduced *Drosophila* transgene in the vicinity of the centromere compared to the euchromatically located transgene [Wallrath and Elgin, 1995]. In mouse cells, Lundgren et al. [2000] compared a λ5 immunoglobulin transgene in centromeric heterochromatin and the endogenous gene in euchromatin. In fibroblast nuclei, where both the transgene and the endogenous gene are silent, the centromeric transgene (present in 4 or 11 copies) in two independent mouse strains was four to five times more resistant to restriction endonuclease cleavage than the endogenous gene.

Recent insights into centromeric heterochromatin have raised questions about histone modification in the pericentromeric regions (centromeric plus juxtacentromeric heterochromatin). Centromeres from budding yeast to metazoans contain homodimers of a variant, centromere-specific histone H3 (CenH3), which is essential for active centromeres [Ahmad and Henikoff, 2002]. CenH3 lacks the canonical histone H3 N-terminal tail and, therefore, is missing H3 K9 hypermethylation, K9 hypoacetylation, and K4 hypomethylation typical of constitutive heterochromatin or silenced euchromatin [Czermin et al., 2001; Jenuwein and Allis, 2001; Noma et al., 2001; Coffee et al., 2002]. Recent findings suggest a dual nature of chromatin in the centromeric region. CenH3-containing nucleosomes residing only in the pre-kinetochore or kinetochore might not be heterochromatic while the adjacent, repeat-rich chromatin containing only canonical H3 is heterochromatic [Sullivan et al., 2001; Ahmad and Henikoff, 2002]. Consistent with the partial substitution of CenH3 for H3 in centromeres of fission yeast and *Drosophila*, these CenH3-rich regions are deficient in HP1 [Ahmad and Henikoff, 2002], whose binding to chromatin often depends on direct interaction with a methylated K9 of histone H3 [Jenuwein and Allis, 2001]. Histone H4 has not been reported to have centromere-specific variants and so, throughout the centromeres, it might retain the N-terminal hypoacetylation associated with heterochromatin.

In human cells, most of the constitutive heterochromatin is found adjacent to the centromeres (juxtacentromeric heterochromatin), in the short arms of the acrocentric chromosomes, in a large portion of Yq, and in the centromeric regions. The human centromeres consist largely, but not exclusively, of tandem repeats of a 171-bp satellite α (Satα) monomer arranged in chromosome-specific, higher-order repeats [Lee et al., 1997]. Only the portion of Satα nucleosomes in the center of human centromeric regions might contain the human CenH3, CENP-A [Ando et al., 2002; Blower et al., 2002]. Typically, around the human Satα-rich centromeric regions are other satellite DNAs [Lee et al., 1997; Schueler et al., 2001]. Such satellite-rich regions are most prominent in chromosomes 1, 9, and 16, where the juxtacentromeric heterochromatin rich in satellite 2 (Sat2 in Chr1 and Chr16) or satellite 3 (Sat3 in Chr9) is much longer than the Satα-containing centromeric region. To elucidate the determinants of constitutive heterochromatin, we have compared histone modifications in Sat2- and Satα-containing heterochromatin with that in unexpressed euchromatin by chromatin immunoprecipitation (ChIP) assays employing semi-quantitative PCR (SQ-PCR) or real-time quantitative PCR (Q-PCR) as well as

by immunocytochemistry and fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Cell lines, Culture Conditions, and Mononuclear Blood Cell Isolation

Six normal human lymphoblastoid cell lines (LCLs) (half were from females) and two skin fibroblast cell strains derived from newborn foreskin were grown under standard conditions. For analysis of mononuclear blood cells, EDTA-treated human peripheral blood samples (half from females) were used to generate a mononuclear cell fraction by density gradient centrifugation (Lymphocyte Separation Medium, Cappel).

Chromatin Immunoprecipitation (ChIP)

About 0.5×10^6 cells were treated with 1% formaldehyde and sonicated in 1 ml of protease inhibitor-containing buffer. Then chromatin was immunoprecipitated essentially according to the manufacturer's specifications. We used a 1:100 dilution of antibody prepared to an H4 N-terminal peptide acetylated at lysines 5, 8, 12, and 16 (H4 Ac Ab, catalog #06-866; all ChIP Abs from Upstate Biotechnology) or at a single lysine residue (H4 Ac K5, H4 Ac K8, H4 Ac K12, or H4 Ac K16 Ab; #07-327, 06-760, 06-761, 06-762); to an H3 peptide acetylated at K9 (H3Ac K9 Ab; #07-352); or to an H3 peptide dimethylated at lysine 4 (H3 Me K4 Ab; #07-030) or 9 (H3 Me K9 Ab; #07-212). The specificity of these antibodies for the four antibody preparations directed to monoacetylated H4 and the antibody to H3 Ac K9 was described [Suka et al., 2001] and for the other antibodies was determined by the manufacturer as follows. For H4 Ac Ab, an acid extract of butyrate-treated HeLa cells gave a single reacting band that coelectrophoresed with acetylated H4. By a dot-blot assay with H4 N-terminal peptides, no reaction for H4 Ac Ab was seen with H3 N-terminal peptide monoacetylated at K9 or K14; a very strong reaction was obtained with the H4 N-terminal peptide mono-acetylated at K8 or K16 or tetra-acetylated at K5, K8, K12, and K16; a moderate reaction was observed with H4 N-terminal peptide mono-acetylated at K12 or K5; and much less reaction was seen with unacetylated H4 N-terminal peptide. Both the H3 Me K4 Ab and H3 Me K9 Ab were shown to react preferentially with the corresponding dimethylated form of the H3 N-terminal peptide in ELISA, and both gave a single band at the expected position upon immunoblot analysis of HeLa acid extracts. Also, this H3 Me K9 Ab reacts preferentially with H3 dimethylated at K9 in immuno-dot blots (T. Jenuwein, unpublished data). In ChIP with these antibodies, the input DNA for comparison to the immunoprecipitates was an aliquot of the supernatant from each centrifuged sonicate. The pre-clearing before addition of antibody and the collection of the immunoprecipitates after incubation with antibody was done for 3 h at 4°C with constant agitation using 60 μl of salmon sperm DNA/protein A-agarose beads added to the 1-ml samples. The immunoprecipitated (IP) DNA samples were purified by proteinase K digestion, phenol-extraction, and ethanol-precipitation and dissolved in 50 μl of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Semi-Quantitative (SQ) PCR

Before SQ-PCR, the DNA was subjected to serial 2-fold dilutions from 2- to 32-fold and 25- to 3,200-fold for the IP and the input DNA, respectively, and 2 μl of the diluted sample was amplified with Taq polymerase (HotStarTaq Master Mix Kit, Qiagen; PTC-100 thermal cycler, MJ Research) using 0.5 μM primers in 25 μl for 32 cycles for single-copy sequences and 28 cycles for satellite DNA (Table I). Ten microliters of the PCR product was electrophoresed in the presence of ethidium bromide and the bands were digitally quantitated under UV transillumination. The average normalized band signals from dilutions displaying approximate inverse proportionality to the dilution factor were used to quantitate IP and input DNA. The undiluted no-antibody control gave either no specific band or, sometimes from amplified satellite DNA, a band having 1% the signal of the IP band.

Real-Time Quantitative PCR

Real-time PCR was performed using SYBR Green dye fluorescence (Applied Biosystems; iCycler, BioRad). The 20-μl reaction mixtures, which contained 350 nM of each primer, 2 μl of undiluted IP DNA or a 1:100 dilution of input DNA, and Taq polymerase (AmplitaqGold polymerase, Applied Biosystems), were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, annealing for 30 s (Table I), and incubation at 72°C for 30 s. The specificity of the PCR

TABLE I. PCR Conditions for ChIP Assays

Type of chromatin	DNA sequence (accession no.)	Amplified region[a]	Primer sequences	Product size (bp)	Annealing temperature (°C)[b]
Inactive euchromatin	*Pre-proinsulin* (V00565) 11p15.5	Within exon 3 (3464–3636) TSP 2186	5′-CCTGCAGAAGCGTGGCATT-3′ 5′-CACAGACGGCACAGCAG-3′	173	58
	G-γ globin (U01317) 11p15.5	Within exon 2 (34756–34950) TSP 34478	5′-TCTACCCATGGACCCAGAGGT-3′ 5′-CCACATGCAGCTTGTCACAGT-3′	195	58
	AFP (M16110) 4q11-22	Within exon 11 (17469–17593) TSP 3141	5′-GTTTCTCGTTGCTTACACAAAG-3′ 5′-AGGCCAATAGTTTGTCCTCACT-3′	125	59
	Albumin (M12523) 4q11-13	Within exon 4 (6071–6209) TSP 1737	5′-GTTGCAACTCTTCGTGAAAC-3′ 5′-TCACATCAACCTCTGGTCTC-3′	139	58
Expressed euchromatin	*GAPDH* (J04038) 12p13	Promoter region, −790 to −498 664–956	5′-CCCAACTTTCCCGCCTCTC-3′ 5′-CAGCCGCCTGGTTCAACTG-3′	293	62
	ADH5 (U10902) 4q21	Within intron 1 (+513 to +658) 171,382–171,527	5′-GCATAATTGAGCCTACGCC-3′ 5′-GCAGAGGTGTTTGTTACGTG-3′	146	59
Constitutive heterochromatin	Chr1 Sat2 (X72623) juxtacentromeric[c]	838–997; higher-order repeat, ∼1.3, 1.8, and 2.3 kb	5′-CATCGATGGAAATGAAAGGAGTC-3′ 5′-ACCATTGGATGATTGCAGTCAA-3′	160	58
	Chr1 Satα (M26919) centromeric[c]	171–306; higher-order repeat, ∼1.9 kb	5′-TCATTCCCACAAACTGCGTTG-3′ 5′-TCCAACGAAGGCCACAAGA-3′	236	54
	Chr4 Satα (M38467) centromeric[c]	162–301; higher order repeat, ∼3 kb	5′-CTGCACTACCTGAAGAGGAC-3′ 5′-GATGGTTCAACACTCTTACA-3	139	52

[a]Positions are given with respect to GenBank numbering except for numbers preceded by a + or −, which denotes the distance from the major transcription start site, TSP. The higher-order repeat sizes for Chr1 Sat2, Chr1 Satα, and Chr4 Satα have been described [Lee et al., 1997; Ando et al., 2002].
[b]The annealing temperatures for both semi-quantitative (SQ) PCR and real-time quantitative (Q) PCR were as listed except that for SQ-PCR with *pre-proinsulin, G-γ globin*, and Chr1 Satα primers for which the temperature was 2°C less.
[c]The specificity of the satellite DNA primers was tested by PCR using a panel of monochromosomal somatic cell rodent-human hybrids. The Chr1 Sat2 primers amplified only Chr1 but the Chr1 Satα primers amplified in addition chromosomes 5, 13, 19, and Y to give the correct size product. The Chr4 Satα primers amplified only Chr4 and ChrY.

products was demonstrated by the presence of one peak in a melting curve and the correct size of the single band seen upon gel electrophoresis of representative PCR products. To quantitate product DNA from the threshold cycle number, a standard curve for each primer-pair and PCR set was generated from serial twofold dilutions of a reference mixture of sonicated human DNA. The slope of the standard curve for each test reaction was -3.3 ± 0.4 and the correlation coefficient was 0.99. For statistical analysis, paired *t*-tests were done.

Immunocytochemistry

Human fibroblasts or mouse A9 cells were grown on slides for 24 h before immuno-staining as follows. The cells were washed with phosphate-buffered saline (PBS), fixed for 10 min with methanol, and permeabilized with PBS/0.5% Triton X-100 for 10 min. The slides were incubated in blocking buffer (120 mM KCl, 20 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 3% bovine serum albumin) for 30 min and then with the following dilutions of the first Ab: H4 Ac K8, 1:200; H3 Me K4, 1:500; HP1α, 1:100 (#05-689); H3 Me K9, 1:1,000 (all from Upstate Biotechnology; catalog numbers as above unless otherwise mentioned); or branched H3 Me K9 peptide (from Thomas Jenuwein, 1:2,000 [Maison et al., 2002]), followed by a conjugated secondary Ab (Alexa Fluor 488, 1:100, Molecular Probes) in blocking buffer for 60 min. The latter H3 Me K9 Ab was raised to a four-branched dimethyl peptide from the H3 N-terminus and has a high affinity for trimethylated H3 K9 using a panel of methylated H3 peptides in a dot-blot analysis ([Maison et al., 2002] and T. Jenuwein, unpublished data). This antibody's in vivo specificity for pericentromeric heterochromatin has been shown to be due to its binding to H3 trimethylated at K9 [Peters et al., 2001]. The slides were then post-fixed in the dark with methanol/acetic acid (3:1) for 10 min and 2% paraformaldehyde for 1 min. DNA was denatured with 0.1 M NaOH for 2.5 min. The slides were immediately rinsed with ice-cold PBS before stepwise dehydration in ethanol. The 1qh-specific probe (CEP chromosome1 satellite 2&3, Vysis) in hybridization buffer was left on the slides at 75°C for 5 min and then overnight at 37°C in a humid chamber. Cells were counterstained with DAPI (4′,6-diamino-2-phenylindole). Deconvolution microscopy (Leica DMRXA microscope, CCD camera; Sensicam QE, Oxford Lasers) was done by collecting images at 0.1- to 0.2-μm steps with quantitation of immuno-FISH signals (the number of voxels) by analyzing images that were deconvolved (Slidebook 4.0; Intelligent Imaging Innovations) using the nearest neighbor algorithm.

RESULTS

Relative Acetylation of Histone H4 by Q-PCR Versus SQ-PCR ChIP Assays

By ChIP assays with H4 Ac Ab, which reacts with H4 N-termini acetylated at one or multiple positions (K8, K16, K5, or K12), we compared H4 acetylation in three different types of chromatin standards by SQ-PCR versus Q-PCR on human LCLs and peripheral blood mononuclear cells (PBMC). These standards were constitutive heterochromatin regions, unexpressed euchromatic gene regions, and promoter or 5′-regions of constitutively expressed genes. For some experiments, DNA in the immunoprecipitate (IP DNA) and sonicate (input DNA; about 0.2–0.6 kb) was quantitated by SQ-PCR done on serial dilutions of each IP and input DNA. Only dilutions that gave approximate proportionality between the relative amounts of template and of signal in the specific PCR product were used to determine the percent immunoprecipitation. Q-PCR was done using the same primers as for SQ-PCR (Table I) continuously monitoring the reaction by SYBR Green fluorescence. To calculate the percent immunoprecipitation, the amount of PCR product from the immunoprecipitate (IP DNA) was divided by that from the input DNA for each set of primers. We compared the percent immunoprecipitation of different sequences from the same samples, which corrects for differences in the amplification with different primer-pairs and in the efficiency of immunoprecipitation from experiment to experiment. This ratio of percentage immunoprecipitation of different DNA sequences in a given batch of cells can be compared to that in another batch of cells and was highly consistent (Fig. 1). In contrast, the percentage precipitation of an individual DNA sequence or just one group of sequences could not be compared from one batch of cells to another (Tables II and III). Using the ratio of percent immunoprecipitation of different groups of DNA sequences, SQ-PCR and Q-PCR analyses gave similar results,

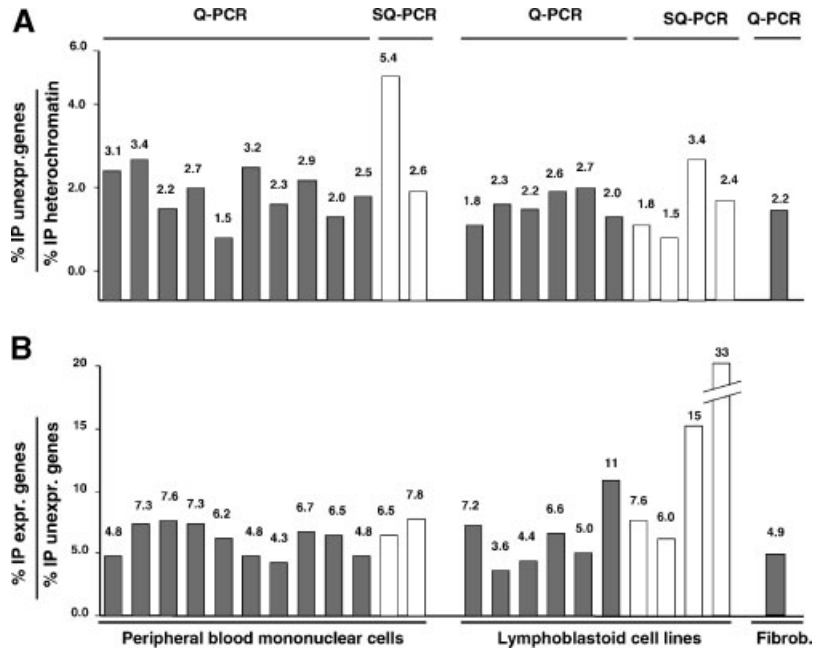

Fig. 1. Comparison of histone H4 acetylation in unexpressed euchromatin, constitutive heterochromatin, and expressed euchromatin in individual PBMC, LCLs, and diploid fibroblast cultures analyzed by ChIP involving Q-PCR (dark bars) and SQ-PCR (light bars). **A**: The average ratios of % immunoprecipitation of unexpressed euchromatin standards to % immunoprecipitation of heterochromatin standards. **B**: The average ratios of % immunoprecipitation of expressed euchromatin standards to % immunoprecipitation of unexpressed euchromatin standards. For Q-PCR, three heterochromatin standards, four unexpressed euchromatin standards, and two expressed euchromatin standards were used. For SQ-PCR, two heterochromatin standards, two unexpressed euchromatin standards, and one expressed euchromatin standard were used. The average ratio for each immunoprecipitation is shown above the bar for blood samples from six individuals, six different control LCLs, and two fibroblast cell strains; some were immunoprecipitated in duplicate. Representative data for the individual euchromatin and heterochromatin standards in ChIP analyses on LCLs and PBMC samples are shown in Tables II and III.

although more outliers were observed in the former analysis (Fig. 1 and Table II).

Immunoprecipitation of Heterochromatin Versus Euchromatin by Antibodies to Acetylated H4 or H3

Promoter or 5′-regions of actively expressed genes typically display high levels of acetylation of core histones [Eberharter and Becker, 2002]. Accordingly, high percentages of the tested constitutively expressed gene regions, that is, the promoter region of the glyceraldehyde phosphate dehydrogenase gene (*GAPDH*) and the first intron of the alcohol dehydrogenase 5 gene (*ADH5*), were immunoprecipitated with the H4 Ac Ab (Table III). Lower percentages of immunoprecipitation were seen for the four analyzed genes (G-γ globin, pre-proinsulin, alpha fetoprotein, and albumin) that should be silent in lymphoblasts, mononucleated leukocytes, and fibroblasts. RT-PCR of RNA from LCLs and PBMC confirmed that there was no detectable expression of these genes (data not shown). Very low percentages of immunoprecipitation were observed using PCR primers for three DNA repeats from the following constitutive heterochromatin regions: centromeric Chr1 Satα, Chr4 Satα, and juxtacentromeric Chr1 Sat2. Uncultured G0 cells (PBMC) and two disparate kinds of cultured cells (LCLs and fibroblast cell strains) gave similar results (Fig. 1). The ratio of immunoprecipitation by H4 Ac Ab of two actively expressed gene regions to four inactive genes was 6.0 ± 1.7 (mean $\pm$ SD) from 19 ChIP/Q-PCR assays (Fig. 1B). The analogous ratio for the percentage immunoprecipitation of four unexpressed genes to three constitutive heterochromatin sequences was 2.5 ± 0.5 for these assays (Fig. 1A). The differences between the extent of immunoprecipitation by H4 Ac Ab of constitutive heterochromatin and unexpressed euchromatin or expressed euchromatin and unexpressed euchromatin were highly significant ($P < 0.001$).

Because the H4 Ac Ab binds to H4 with more than one acetyl group as well as to singly

TABLE II. ChIP Analysis of Histone H4 Acetylation With Different Antibodies on Lymphoblastoid Cell Lines by SQ-PCR[a]

		Percent immunoprecipitation[b]					Relative immunoprecipitation of two types of sequences	
		Heterochromatin		Unexpressed euchromatin		Expressed euchromatin		
Ab to	LCL ID	Chr1 Satα	Chr1 Sat2	*G-γ globin*	*Pro-insulin*	*GAPDH*	Expressed euchromatin versus unexpressed euchromatin[c]	Unexpressed euchromatin versus heterochromatin[d]
H4 Ac	1	6.0	7.6	11	13	91	7.6	1.8
	2	5.4	4.8	9.6	5.2	44	6.0	1.5
	3	0.20	0.14	0.39	0.44	14	33	2.4
	Average (SD)						15 (15)	1.9 (0.5)
H4 Ac K5	4	0.12	0.25	0.25	0.41	0.17	0.5	1.8
H4 Ac K8	4	0.33	0.35	0.50	0.67	7.5	12.8	1.7
	1	11	6.4	12	12	37	3.0	1.4
	2	5.4	3.6	9.6	5.3	22	3.0	1.7
	Average (SD)						6.3 (5)	1.6 (0.2)
H4 Ac K12	1	8.4	5.6	7.2	9.1	14	1.7	1.2
	2	3.5	3.9	4.9	4.8	10	2.1	1.3
	Average (SD)						1.9 (0.3)	1.3 (0.1)
H4 Ac K16	4	0.87	1.9	1.7	1.5	2.2	1.4	1.1
	1	5.1	5.3	4.0	6.1	4.6	0.9	1.0
	2	1.3	1.1	1.0	ND	0.6	0.6	0.8
	Average (SD)						1.0 (0.4)	1.0 (0.2)

[a]Semi-quantitative (SQ) PCR was done on four to eight serial dilutions of each DNA sample (input or IP DNA). The LCL 1, 2, and 4 samples analyzed by SQ-PCR were immunoprecipitated the same day and so can be compared to each other. The H4 Ac Ab reacts with H4 acetylated at K5, K8, K12, or K16.
[b]Percent immunoprecipitation, 100× the amount of the DNA sequence in the immunoprecipitate divided by the amount in input DNA determined by PCR. The percent immunoprecipitation was analyzed for centromeric heterochromatin (Chr1 Satα primers), juxtacentromeric heterochromatin (Chr1 Sat2 primers), intragenic sequences in the indicated unexpressed (in lymphoid cells) genes, and for a promoter sequence in a constitutively expressed gene (*GAPDH*).
[c]The percent immunoprecipitated (IP) expressed euchromatic sequence divided by the average % IP unexpressed euchromatic sequences. The average ratios for each antibody and the standard deviation are given.
[d]The mean % IP unexpressed euchromatic sequences divided by the mean % IP heterochromatic sequences. For the H4 Ac Ab, which can immunoprecipitate various singly or multiply acetylated forms of H4, many more samples were analyzed (Fig. 1) and the overall average ratio of % IP unexpressed euchromatin divided by % IP heterochromatin was 2.5 for Q-PCR amplified samples.

TABLE III. ChIP Analysis of Histone H3 and H4 Acetylation With Different Antibodies on Peripheral Blood Mononuclear Cells by Q-PCR[a]

		Percent immunoprecipitation								Relative immunoprecipitation of two types of sequences	
		Heterochromatin			Unexpressed euchromatin		Expressed euchromatin			Expressed euchromatin versus unexpressed euchromatin	Unexpressed euchromatin versus heterochromatin
Ab to	ID	Chr1 Satα	Chr4 Satα	Chr1 Sat 2	*G-γ globin*	*Pro-insulin*	*AFP*	*Alb.*	*GAPDH*	*ADH5*	
H4 Ac	A	0.28	0.32	0.19	0.73	1.2	0.63	0.71	3.7	4.2	4.8	3.2
	B	0.34	0.48	0.56	1.2	1.5	0.93	0.75	4.2	5.1	4.3	2.3
H4 Ac K5	A	0.16	0.08	0.06	0.20	0.14	0.16	0.18	0.23	0.47	2.1	1.7
	B	0.24	0.13	0.12	0.26	0.15	0.20	0.16	0.38	0.37	2.0	1.2
H4 Ac K8	A	0.19	0.18	0.14	0.30	0.50	0.26	0.34	2.8	3.2	8.6	2.1
	B	0.22	0.18	0.08	0.33	0.30	0.25	0.16	1.2	1.1	4.3	1.6
H3 Ac K9	C	0.51	0.65	0.59	0.70	0.69	0.99	0.92	1.9	2.1	2.5	1.4
	D	0.84	1.2	1.2	0.96	1.1	1.3	0.83	2.7	2.3	2.4	1.0

[a]Analysis by real-time PCR was done in duplicate on each DNA sample and the percent immunoprecipitation is the average of duplicates; almost all the duplicates agreed within ±20%. *AFP*, *Alb*, *ADH5*, and Chr1 Satα refer to two additional unexpressed gene regions, one constitutively expressed gene region, and one centromeric heterochromatin region, respectively, not given in Table II. The PBMC samples from normal subjects A and B were prepared on the same day so that the same standard PCR curves for calibrating DNA concentrations were used and the relative amount of immunoprecipitate formed with different antibodies can be compared.

acetylated N-terminal tails of H4, and transcriptionally active genes seem to have multiple acetyl groups on H4 N-terminal tails [Grandjean et al., 2001; Myers et al., 2001], an antibody to a specific monoacetylated form of H4 might be a better identifier to distinguish euchromatin from heterochromatin. Therefore, we compared unexpressed euchromatin regions and constitutive heterochromatin in ChIP assays with four antibody preparations that were specific for monoacetylation at K5, K8, K12, or K16 of histone H4 [Suka et al., 2001]. Given the central role ascribed to di- or trimethylation of H3 K9 in recruiting HP1 proteins to heterochromatic regions and the opposite functionality of H3-K9 methylation and H3-K9 acetylation [Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002], we also tested an antibody for H3 Ac K9. The H4 Ac K5, H4 Ac K8, and H3 Ac K9 Abs, like the H4 Ac Ab, were designated as ChIP grade by the supplier. All antibodies except for H4 Ac K5 gave considerable amounts of immunoprecipitation of active gene regions (Tables II and III). H4 Ac Ab gave the largest, albeit a moderate, difference in the extent of immunoprecipitation of unexpressed euchromatin and constitutive heterochromatin (Tables II and III).

Immunoprecipitation of Heterochromatin Versus Euchromatin by Antibodies to Methylated H3

Because available antibodies to HP1α and HP1β gave little or no immunoprecipitate even from heterochromatin (data not shown), we used H3-K9 methylation as an indicator of HP1-rich heterochromatin [Li et al., 2002] in ChIP assays. The ChIP-grade H3 Me K9 Ab, which is specific for dimethylation at K9, consistently immunoprecipitated a higher percentage of constitutive heterochromatin than of expressed euchromatin (Table IV). However, it gave no reproducible difference in the levels of IP unexpressed euchromatin and IP constitutive heterochromatin and was not very efficient at precipitating even constitutive heterochromatin. The same batches of sonicated chromatin, which showed low levels of immunoprecipitation of the expressed euchromatin standards by this H3 Me K9 Ab, displayed relatively high levels of immunoprecipitation of these standards by H3 Me K4 Ab, which is specific for dimethylation at K4 (Table IV) and with the H4 Ac Ab (Table II). Although dimethylation at H3 K9 in a chromatin region

TABLE IV. ChIP Analysis of Histone H3 Methylation in LCLs and PBMC[a]

			Percent immunoprecipitation								Relative immunoprecipitation of two types of sequences	
			Heterochromatin standard		Unexpressed euchromatin				Expressed euchromatin			
Ab to	Cell type and ID	PCR: SQ or Q	Chr1 Satα	Chr1 Sat2	*G-γ globin*	*Pro-insulin*	*AFP*	*Alb.*	*GAPDH*	*ADH5*	Expressed euchromatin versus unexpressed euchromatin	Unexpressed euchromatin versus heterochromatin
H3 Me K9	LCL 1	SQ	0.17	0.30	0.31	0.17	—	—	0	—	0	1.0
	LCL 1	SQ	0.32	0.64	0.25	0.11	—	—	0	—	0	0.4
	LCL 2	SQ	1.3	1.2	1.2	0.27	—	—	0	—	0	0.6
	LCL 3	SQ	0.12	0.22	0.39	0.11	–	—	0	—	0	1.5
	LCL 4	SQ	0.87	1.6	0.75	0.67	—	—	0	—	0	0.6
	LCL 1	Q	0.33	0.40	0.27	0.11	—	—	0.04	—	0.2[b]	0.5[b]
	LCL 2	Q	0.33	0.38	0.60	0.14	—	—	0.05	—	0.1	1.0
	PBMC C	Q	1.2	0.52	0.94	0.13	0.95	1.3	0.12	0.58	0.4	1.1
	PBMC D	Q	1.1	1.1	1.3	0.12	1.3	1.2	0.11	0.36	0.2	0.7
	Fibrob. 1	Q	1.0	0.72	1.8	0.26	0.67	1.5	0.35	0.04	0.2	1.2
H3 Me K4	PBMC C	Q	0.16	0.07	0.31	0.40	0.21	0.32	9.3	14	38[b]	2.1[b]
	PBMC D	Q	0.27	0.34	0.45	0.45	0.33	0.34	14	9.5	30	1.1
	PBMC E	Q	0.17	0.11	0.23	0.26	0.14	0.12	7.4	7.7	40	1.4
	PBMC A	Q	0.16	0.14	0.21	0.08	0.09	0.08	3.4	3.3	29	0.9
	Fibrob. 1	Q	0.12	0.16	0.55	0.13	—	0.50	15	13	36	2.2
	Fibrob. 2	Q	0.10	0.14	0.14	0.08	0.77	0.23	1.3	6.9	13	4.2
	Fibrob. 3	Q	0.07	0.04	0.24	0.18	1.4	0.36	3.3	4.6	7.4	5.8

[a]PBMC, peripheral blood mononuclear cells; fibrob., fibroblasts; other abbreviations are as described in Tables II and III.
[b]For the samples analyzed by Q-PCR, one extra heterochromatic standard, Chr4 Satα, was used; those data are not shown but were included in the calculation of the relative immunoprecipitation of unexpressed euchromatin and constitutive heterochromatin. As in Table III, similar results were obtained from Chr4 Satα, Chr1 Satα, and Chr1 Sat2.

is strongly associated with its lack of transcription, methylation at H3 K4 is linked to transcriptional activity [Noma et al., 2001]. As expected, H3 Me K4 Ab showed a very strong preference for immunoprecipitation of active euchromatin relative to unexpressed euchromatin. However, this antibody did not give consistent differences in the extent of immunoprecipitation of inactive euchromatin versus constitutive heterochromatin (Table IV).

Immunocytochemical Analysis of Histone Modification in Heterochromatin Versus Euchromatin

We also compared histone modification in heterochromatin and euchromatin in primary human fibroblasts by immunocytochemistry using the same antibody preparations as for ChIP assays. We found that H4 Ac K8 and H3 Me K4 antibodies were largely excluded from bulk constitutive heterochromatin and from the Chr1 Sat2 region at 1qh (Fig. 2). We quantitated the extent of colocalization of antibody signals and DAPI-bright loci, which are indicative of constitutive heterochromatin, by deconvolution microscopy on randomly chosen cells analyzing the signals in 8–12 planes per cell. Similar results were obtained from the different planes as well as from different cells. We found only $16 \pm 3\%$ and $12 \pm 1\%$ colocalization of the antibody signal and the DAPI-bright fluorescent loci for the H4 Ac K8 Ab and the H3 Me K4 Ab, respectively (standard deviation given for comparison of results from three or five different cells). Also, as previously reported, there was a broad granular staining throughout most of the nucleus in cells incubated with the H3 Me K9 Ab that was specific for H3 dimethylated at K9 (linear H3 Me K9, Fig. 2) [Maison et al., 2002]. However, as observed in a previous study [Maison et al., 2002], there was appreciable colocalization ($40 \pm 5\%$ in 11 cells) of DAPI-bright material and an antibody to a branched form of the H3 Me K9 peptide (Fig. 2) that is thought to predominantly recognize H3 Me_3 K9 [Peters et al., 2001]. Nonetheless, we consistently found that about 60% of the DAPI-bright material gave no colocalization with this antibody in human fibroblasts. This was most clearly seen by deconvolution microscopy, where the cell is viewed in sections, rather than by epifluorescence microscopy, where the whole cell is viewed at once. Murine A9 cells showed much more colocalization ($86 \pm 4\%$) of this antibody and the DAPI-bright material (Fig. 2B). The difference between colocalization of this antibody with DAPI-bright material in human and mouse cells was highly significant ($P < 0.0001$; unpaired t-test). Similar results with human fibroblasts were obtained upon immuno-FISH using an Ab to HP1α [Minc et al., 1999] as with the antibody to the branched form of the H3 Me K9 peptide (Fig. 2B). This is consistent with the finding that the recruitment of HP1α to heterochromatin has been shown to be often dependent on methylation of H3 K9 [Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002].

DISCUSSION

By quantitative ChIP assays, we assessed histone modification in constitutive, pericentromeric heterochromatin and unexpressed euchromatin in several types of human cells to test whether there are consistent differences that might contribute to the maintenance of the structure of constitutive heterochromatin. It has been proposed that the highly condensed state of pericentromeric heterochromatin is partially due to HP1 binding in response to changes in modification of the tails of the core histones [Belyaeva et al., 1993; Jenuwein, 2001]. H3 K9 hypoacetylation, often coupled with general hypoacetylation of the N-terminal tail of H4, is necessary for H3-K9 methylation. H3-K9 methylation, in turn, is frequently required for HP1 binding to chromatin [Eberharter and Becker, 2002; Li et al., 2002], and the resulting HP1 multimerization might foster heterochromatinization [Wang et al., 2000]. The specific interaction of H3 Me K9 with HP1 has been observed with an N-terminal H3 peptide di- or trimethylated at K9 and a chromodomain peptide from murine HP1β [Nielsen et al., 2002].

Our results from ChIP assays on human LCLs, PBMC, and diploid fibroblasts argue against the hypothesis that a high level of dimethylation of histone H3 at K9 is a defining feature of constitutive heterochromatin in eucaryotes [Bailis and Forsburg, 2002; Cowell et al., 2002], consistent with a recent immunocytochemistry study of murine embryonal stem cells [Peters et al., 2003]. We found no significant difference in the extent of H3 K9 methylation between heterochromatin and unexpressed euchromatin standards in ten ChIP assays using an antibody specific for H3

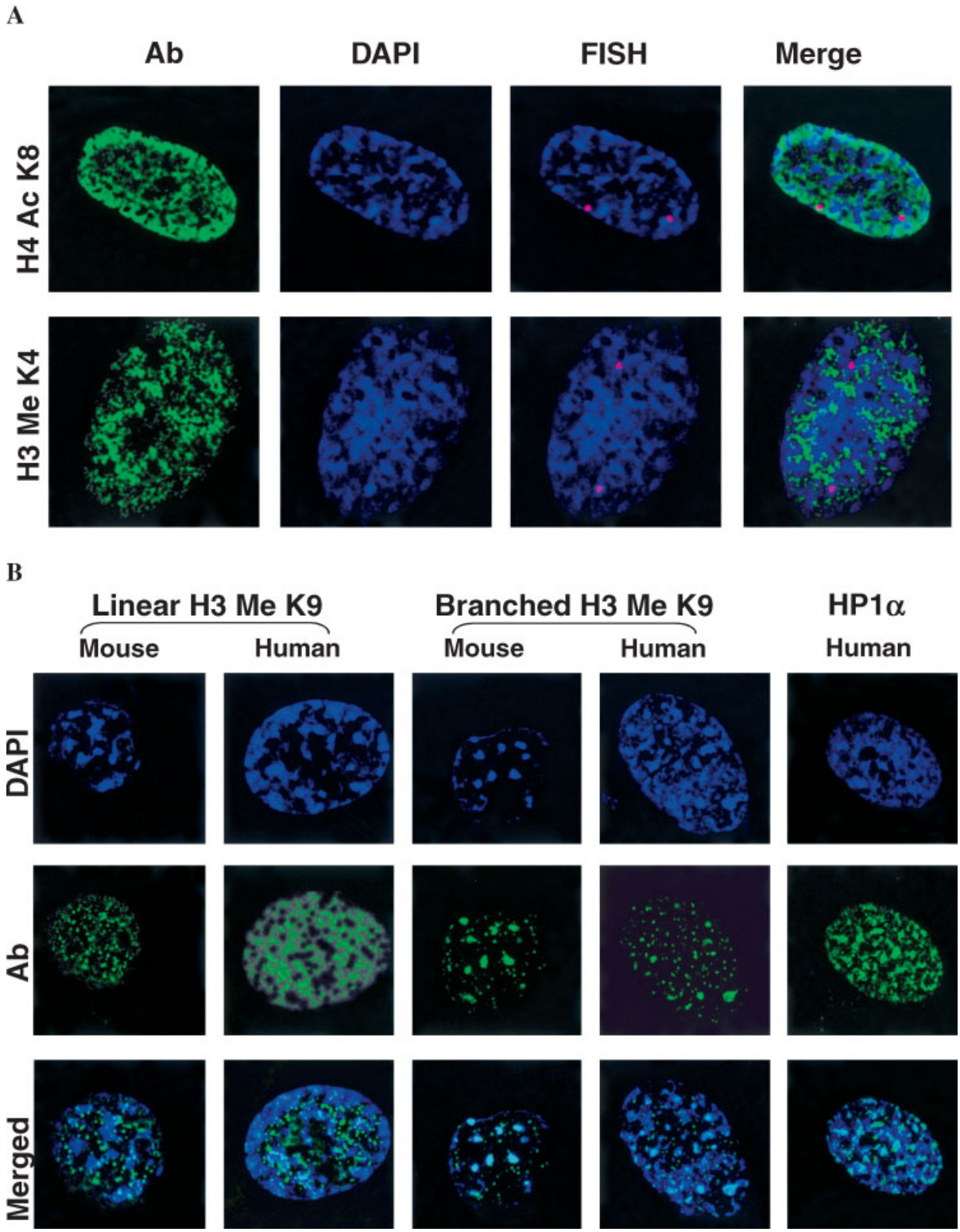

Fig. 2. Immunocytochemical analysis of histone methylation in constitutive heterochromatin compared to euchromatin analyzed by deconvolution microscopy. **A**: Immuno-FISH with H4 Ac K8 Ab or H3 Me K4 Ab on diploid human fibroblasts. **B**: Immunocytochemistry with H3 Me K9 Abs or HP1α Ab on diploid human fibroblasts and a mouse cell line. Foci of constitutive heterochromatin are seen as bright blue, DAPI-stained areas. The red FISH stain for 1qh satellite DNA appears purple because it overlaps the heterochromatic DAPI-bright foci. The indirect antibody stain (green) becomes light blue when it overlaps the DAPI-bright material. Note in the last two columns of (B) that in human cells, even within an individual DAPI-bright focus which shows some strong binding of the antibody to branched H3 Me K9 peptide or to HP1α, much of the bright blue focus often does not have superimposed green signal.

dimethylated at K9. This antibody, as expected [Litt et al., 2001; Coffee et al., 2002], gave lower levels of immunoprecipitation of expressed than unexpressed euchromatin standards (Table IV). Immunocytochemistry further elucidated the relationship between H3-K9 methylation and euchromatin versus constitutive heterochromatin. As previously reported [Maison et al., 2002], immunocytochemical analysis showed that the standard antibody for H3-K9 dimethylation had no specificity for DAPI-bright regions, which correspond to constitutive heterochromatin (Fig. 2). We also used an antibody raised to an artificially branched K9-dimethylated N-terminal H3 peptide, which has a preference for H3 trimethylated at K9 [Peters et al., 2001]. There was partial colocalization of this antibody with constitutive heterochromatin in the nuclei of human fibroblasts and, to a much greater extent, mouse cells (Fig. 2). Importantly, about 60% of the chromatin in heterochromatic foci in human cells did not appreciably react with this antibody. In contrast, in mouse cells, only about 15% of DAPI-bright material did not colocalize with this antibody's signal (Fig. 2) indicating that there was no technical problem with heterochromatin reacting cytochemically with this antibody. Moreover, our finding that HP1α Ab immunostaining also showed only very partial overlap with heterochromatic foci in human cells is consistent with the H3 K9 Ab results because H3 K9 trimethylation recruits HP1α to heterochromatin [Peters et al., 2001, 2003; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002]. We conclude that high levels of H3 trimethylated or dimethylated at K9 do not seem to be generally necessary for the maintenance of constitutive heterochromatin in mammalian cells.

While histone methylation in constitutive heterochromatin had not been previously compared with that in unexpressed euchromatin in ChIP assays on mammalian cells, analogous studies had been described for histone acetylation. It was reported that immunoprecipitates analyzed by slot-blot hybridization show varying extents of hypoacetylation of heterochromatin relative to either unexpressed or expressed euchromatin [O'Neill and Turner, 1995; Johnson et al., 1998]. O'Neill and Turner [1995] examined histone acetylation in a human promyeloid cell line (HL60) using antibodies that recognize H4 acetylated at 1, 2-4, or 3-4 acetyl groups on the N-terminal tail of histone H4. Their hybridization probes were Sat3 (a simplified oligonucleotide form of the 9qh and Yqh repeat), centromeric Satα (an oligonucleotide partially homologous to part of the 171-bp consensus sequence), and coding regions of two constitutively expressed and three unexpressed genes. Compared with either unexpressed or expressed euchromatic regions, an average of 2–16- and 12–80-fold less Satα and Sat3, respectively, was found in the immunoprecipitates obtained with the different acetylated H4-specific antibodies. Using a human LCL and antibodies to various monoacetylated forms of H4, the same group observed that about 1.5–4 times less Satα and 2.5–9 times less Sat3 immunoprecipitated than for either silent or expressed gene regions in euchromatin [Johnson et al., 1998]. Surprisingly, in those studies, there were no expression-related differences in the extent of immunoprecipitation between the intragenic regions from five unexpressed genes and three constitutively expressed genes. In the present study, we examined two centromeric Satα sequences, one juxtacentromeric Sat2 sequence and six euchromatic sequences (five of which are different from those examined by O'Neill and colleagues) in human LCLs, PBMC, and diploid fibroblasts. We found that the average ratios of percent immunoprecipitation of unexpressed euchromatin to that of constitutive heterochromatin were only 2.5 and 1.7 with H4 Ac Ab and H4 Ac K8 Ab, and we saw no cell-type specific differences. Of the eight antibodies to modified H3 or H4 tails that we tested, H4 Ac Ab gave the most consistent differences ($P < 0.001$) between the relative extent of immunoprecipitation of unexpressed euchromatin and constitutive heterochromatin (Fig. 1).

In the present study, we compared the ability of centromeric Satα repeats and juxtacentromeric Sat2 (1qh) repeats to be immunoprecipitated by antibodies to specific modifications that can be introduced in H3 but not into analogous positions in the centromere-specific H3 variant CENP-A [Ahmad and Henikoff, 2002; Ando et al., 2002]. The 1qh region should not have any CENP-A nucleosomes because CENP-A is confined to centromeric regions. There were no significant differences in the percentage immunoprecipitation of these two types of satellite DNA-rich sequences by antibodies to H3 Me K9, H3 Me K4, or to acetylated H4. These results suggest that most of the Satα-rich chromatin in

the centromeric region contains conventional H3 rather than CENP-A and is heterochromatic, like juxtacentromeric heterochromatin.

The histone code [Jenuwein, 2001; Cowell et al., 2002; Richards and Elgin, 2002] implies that the difference between the higher-order structure of unexpressed euchromatin and constitutive heterochromatin is established by differential modification of the core histones, especially at their N-terminal tails. Our ChIP data show that the differences in H4 acetylation between constitutive heterochromatin and unexpressed euchromatin were not large, although they were consistent, and, so, probably cannot explain the higher level of condensation of pericentromeric heterochromatin than of unexpressed euchromatin. Also by immunocytochemistry, human cells had a large fraction of heterochromatic foci that did not show enrichment for antibodies binding to HP1α or H3 trimethylated at K9. HP1α and HP1β have been proposed to play a major role in responding to the histone code for constitutive heterochromatin and are largely, but not completely, localized to heterochromatic foci in normal murine cells [Melcher et al., 2000; Maison et al., 2002]. When there is dispersion of HP1α or HP1β throughout the nuclei of murine cells or a large decrease in H3 K9 methylation due to trichostatin A or RNase treatment of cells, there is no loss of heterochromatic foci [Melcher et al., 2000; Maison et al., 2002]. Also, cells from double-null *Suv39h1*/*Suv39h2* mice that have a large decrease in trimethylation of H3 K9 in heterochromatic foci [Peters et al., 2001], still display normal DAPI-bright heterochromatic foci [Melcher et al., 2000; Maison et al., 2002]. Lastly, immunocytochemical analysis of *Arabidopsis* nuclei from leaves or roots of wild-type plants revealed that a small but significant percentage of heterochromatic foci displayed much more H4 K16 acetylation than was seen in the euchromatic regions [Jasencakova et al., 2003]. Moreover, in analogous cells from a mutant with a knockout of the *kyp* H3 K9 methyltransferase gene and, therefore, a strong reduction in the H3 Me K9 signal in heterochromatic foci, there was no decrease in the size, shape, or number of these foci. Our results and those from the above-mentioned studies suggest that although H3 K9 trimethylation, H3 and H4 N-terminal hypoacetylation, and preferential binding of HP1α clearly have important roles to play in mammalian pericentromeric heterochromatin, for example, in chromosome segregation [Peters et al., 2001], they are not essential for determining the structure of constitutive heterochromatin. Other factors, such as H3-K27 trimethylation [Peters et al., 2003], might play alternative roles in establishing heterochromatin.

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